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NORTH WESTERN ADELAIDE

HEALTH SERVICE

PROVISIONAL SPECIFICATION

Invention Title:

Methods For Regulating Cancer.

The invention is described in the following statement

141375113

Methods For Regulating Cancer

Field of the invention:

The present invention relates to methods for regulating cancer. In particular, the invention relates to methods for inhibiting cancerous growth of a cell. The invention also provides methods for preventing or treating cancer. The invention also relates to methods of identifying agents that can inhibit cancerous growth of a cell.

Background of the invention:

Cancer describes a range of diseases, which result from dysregulated growth of cells of the body. Malignant cancers may develop from this dysregulated growth and subsequently spread around the body *via* the bloodstream or the lymphatic system, a process known as metastasis. Malignant tumours of epithelial tissues are the most common form of cancer and are responsible for the majority of cancer–related deaths in western industrialised countries. According to the Australian Institute of Health and Welfare (AIHW), on average one in three men and one in four women will develop cancer before the age of 75 years (1). In men the most common cancers are prostate, bowel and lung and in women, breast, bowel and melanoma. Identification of genes expressed specifically in tumour tissues and not in normal tissues, and analysis of their functions are useful for identifying new targets for cancer therapy.

Several genes have been implicated in various cancers. For instance, oncogenes are known to code for receptors for cellular growth factor such as epidermal growth factor. The *ras* gene is an oncogene that is believed to be responsible for up to 90% of all human pancreatic cancer, 50% of human colon cancers, 40% of lung cancers, and 30% of leukemias. Mutated oncogenes can become cancer-causing genes. Such mutated oncogenes code for proteins such as protein kinases and protein phosphorylating enzymes that trigger uncontrolled cell growth. *EphB4* is a recently identified member of the largest known family of receptor protein tyrosine kinases. Eph receptor family members have been identified to be involved in many cellular processes including neural development, angiogenesis and vascular network assembly (2-5). As a result of interactions with their ligands, the ephrins, they mediate contact-dependent cell interactions, which regulate cell functions such as contact inhibition, cytoskeletal organisation and cell migration (6, 7).

Although a number of anti-cancer agents including growth inhibitory molecules such as cytoxic compounds have been developed in an attempt to treat cancer, there still remains a need for providing effective methods for regulating cancer.

Summary of the invention:

The present invention is based on the surprising finding that an antibody that can bind to a particular region of the EphB4 protein can advantageously inhibit cancerous growth in a cancer cell by causing cell death of the cancer cell.

Therefore, in a first aspect the present invention provides a method for inhibiting cancerous growth of a cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 16 to 539 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. In a second aspect the present invention also provides a method for inducing cell death of a cancer cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 16 to 539 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto.

In a third aspect the present invention provides a method for treating or preventing cancer in a subject, the method comprising administering to the subject an effective amount of at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 16 to 539 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. In another aspect of the invention there is provided a method of identifying an agent which inhibits cancerous growth of a cell, the method comprising assessing the ability of the agent to bind to an EphB4 polypeptide within the region of residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto.

The present invention also provides an agent identified by the method described above.

Brief description of the figures:

Figure 1 shows immunohistochemical localisation of *EphB4* expression in three different colon cancers and matched normal mucosa. The dark stain from the biotinylated secondary antibody indicates the EphB4 protein. Nuclei are stained with Harris haematoxylin. High power (100X) magnification images of three different adenocarcinomas (well differentiated, moderately well differentiated and poorly differentiated) and their matched normal mucosa are shown. Strong staining of the tumour tissue and very weak, diffuse staining of normal tissue was evident for each sample set. There was no cross-reactivity with the secondary antibody alone (result not shown).

Figure 2 shows relative RT-PCR comparing expression of *EphB4* (1187 bp) and internal 18S rRNA (489 bp) in five tumour (T) / normal (N) pairs. C1 – colon cancer cell line LIM2405, C2 – colon cancer cell line SW480, RT-RT negative control, P – PCR negative control, M – pUC19/*Hpa*II marker.

Figure 3 shows a graph showing effect of increased doses of EphB4 antibody on growth of MCF-7 cells *in vitro* after 48 hours.

Figure 4 shows a graph showing percentage viability of breast cancer cells after 65 h treatment with five different EphB4 antibodies – Swiss (gift from Dr Andrew Ziemiecki, University of Bern), N – Santa Cruz polyclonal N-terminal specific, C - Santa Cruz polyclonal C-terminal specific, H – Santa Cruz polyclonal internal region (extracellular motifs) batch #2, H (old) – Santa Cruz polyclonal internal region (extracellular motifs) batch #1. Cells were treated with 1/100 dilution of stock antibody (200 µg/ml), then stained with trypan blue (stains dead cells). Ratios of unstained (viable) to stained (unviable) were calculated for four different aliquots of each treatment. Control – no antibody added. CLM – complement limited medium. FCS – 10% Fetal calf serum added to medium.

Figure 5 shows the amino acid sequence of SEQ ID NO:1. SEQ ID NO:1 shows *Homo sapiens* Ephrin type-B receptor 4 (EphB4) sequence.

Detailed description of the invention:

In a first aspect the present invention provides a method for inhibiting cancerous growth of a cell, the method comprising contacting the cell with at least one

antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 16 to 539 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. In a preferred embodiment of the invention the epitope is located within 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto.

The antibody or an antigen-binding portion thereof preferably specifically binds to a polypeptide having a sequence consisting of residues 16 to 539 of SEQ ID NO: 1, more preferably residues 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the antibody or an antigen-binding portion thereof is a polyclonal or monoclonal antibody.

The method preferably results in the death of the cell.

In a second aspect the present invention also provides a method for inducing cell death of a cancer cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 16 to 539 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto.

In a third aspect the present invention provides a method for treating or preventing cancer in a subject, the method comprising administering to the subject an effective amount of at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 16 to 539 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto.

The cancer is preferably selected from the group consisting of breast cancer, prostate cancer, bowel cancer, colon cancer, ovarian cancer, lung cancer, melanoma, lymphoma and leukemia. The method preferably results in the death of a cancer cell in the subject.

In another aspect of the invention there is provided a method of identifying an agent which inhibits cancerous growth of a cell, the method comprising assessing the ability of the agent to bind to an EphB4 polypeptide within the region of 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto.

In a preferred embodiment of the invention, the agent binds to an epitope

contained within residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the epitope is located within residues 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto.

The present invention also provides an agent identified by the method described above.

In the present specification the term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments. The term "epitope" refers to an antigenic determinant region of a polypeptide that is recognized by an antibody or an antigen binding portion thereof.

Antibodies refer to immunoglobulin molecules comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (HCVR or VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR, CDR1, FR2, CDR2, FR3, CDR3, FR4.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (I) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody; (v) a dAb fragment (8) which

consists of a VH domain, or a VL domain (9); and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv)(10), (11).. Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies or triabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (12, 13).

Preferably, the antibody isEphB4 (H-200) rabbit polyclonal Ig G antibody, Santa Cruz Biotechnology, Santa Cruz, California.. Therefore, suitable antibodies useful in the methods of the present invention can include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, and a Fab expression library.

More preferably, the antibody is a monoclonal antibody or fragment thereof and, particularly, is selected from monoclonal antibodies or fragments thereof which bind to an antigenic determinant within residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method, isolated from phage antibody libraries, or may be made by recombinant DNA methods. Such

techniques include, but are not restricted to, the hybridoma technique(14), the trioma technique, the human B-cell hybridoma technique (15), and the EBV hybridoma technique to produce human monoclonal antibodies (16). Furthermore, suitable human antibodies may be produced using transgenic animals using for example techniques described in *Oncology* 29 (Supp 4) 47-50 (2002). The antibodies of the present invention may also be obtained from commercial sources.

Various procedures known in the art may also be used for the production of polyclonal antibodies which can bind to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. For production of the antibodies, various host animals can be immunized by injection with a EphB4 protein or a polypeptide comprising residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. Suitable host animals include, but are not limited to, rabbits, mice, rats, etc. Various adjuvants can be used to increase the immunological response, depending on the host species, and include, but are not limited to, Freud's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, and potentially useful human adjuvants such as *Bacillus Calmette-Guerin* (BCG) and *Corynebacterium parvum*. Antibodies and antibody fragments may be produced in large amounts by standard techniques (eg in either tissue culture or serum free using a fermenter) and purified using affinity columns such as protein A (eg for murine Mabs), Protein G (eg for rat Mabs) or MEP HYPERCEL (eg for IgM and IgG Mabs).

Suitable antibodies may include antibody fragments that include an antigen-binding portion that can bind to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. The antigen-binding portion of an antibody preferably includes idiotypes of residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. Such antibody fragments can be generated by techniques known in the art. For example, such fragments include, but are not limited to, the F (ab')2 fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments that can be generated by reducing the disulfide bridges of the F (ab')2 fragment, the Fab fragments that can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments. In a further technique, recombinant antibodies specific to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90%

identical thereto, can be engineered and ectopically expressed in a wide variety of cell types. . .

The antibodies used in the present methods can include "humanized" forms of non-human (eg., murine) antibodies that are immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab').sub.2 or other antigen-binding subsequences of antibodies) which contain minimal amino acid residues derived from a non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human FR residues. Furthermore, a humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications can be made to further refine and optimize antibody performance.

The term "EphB4 protein" as used herein is taken to include full length EphB4 protein or a polypeptide fragment that comprises residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. A EphB4 variant protein may been modified at the amino acid level and may include additions or deletions or replacements of amino acids which do not affect the functionality of the protein, such as conservative amino acid subsitutions. An EphB4 protein may also include a truncated EphB4 protein. An EphB4 protein may be natural or recombinant. The EphB4 protein may be from any animal species, preferably the EphB4 protein is human.

An antibody or an antigen-binding portion thereof that is suitable for the methods of the present invention, preferably can inhibit cancerous growth of a cell by inhibiting the activity of an EphB4 protein. In the specification the term "cancerous growth" is taken to refer to abnormal and uncontrollable division and growth of a cell. Typically such a cell is identified as a cancer cell that may be able to invade and disrupt other tissues and has the potential to spread to other areas of the body. Cancerous growth of a cell can lead to the formation of a tumor that may be benign or malignant.

In the specification the term "cell(s)" is taken to include any cells. Preferably, the cells are derived from a mammalian species, such as, but not limited to, human, mice, bovine, sheep or domestic animals. It is preferred that the cells are selected from the group including, but not limited to, prostate cells, breast cells, colon cells, fibroblasts, epidermal cells, placental, liver, kidney, pancreas, heart, neural or muscle cells, or cancer or tumor cells. The cells may be normal cells, diseased cells, adult cells or embryonic cells. The cells may be single cells, cultured cells or part of a tissue. The cells may be genetically modified recombinant cells, such as a transgenic cell. Preferably, the cells express EphB4. The cells may be part of a whole animal. The cells may also be derived from a cell line. Preferably, the cells are from cell lines derived from, but not limited to, prostate, breast, colon or ovary. More preferably, the cell lines are selected from the group consisting of breast cancer cell line MCF-7 and colon cancer cell line SW480.

The antibody or an antigen-binding portion thereof of the present invention preferably can inhibit cancerous growth of one or more of cancer cells selected from the group consisting of breast cancer cells, prostate cancer cells, bowel cancer cells, colon cancer cells, ovarian cancer cells, lung cancer cells, melanoma cells, lymphoma cells and leukemia cells.

The antibody or an antigen-binding portion thereof preferably specifically binds to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, more preferably residues 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. In a preferred embodiment of the invention, at least one antibody or an antigen-binding portion thereof specifically binds to an epitope contained within residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. The term "specifically binds" in this specification, is to be understood to refer to binding characteristics of an antibody or an antigen-binding portion thereof which binds exclusively to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, more preferably residues 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. The antibody or an antigen-binding portion thereof is preferably a polyclonal or monoclonal antibody that specifically binds to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, more preferably residues 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody is Santa Cruz, polyclonal H-200 antibody.

The present invention provides a method for inhibiting cancerous growth of a cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 16 to 539 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto.

Preferably, at least one antibody or an antigen-binding portion thereof binds to a polypeptide having a sequence comprising residues 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or an antigen-binding portion thereof binds to a EphB4 protein having a sequence comprising residues 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto.

The phrase "inhibiting cancerous growth of a cell" as used herein is taken to mean that cancerous growth of the cell is substantially reduced or prevented. In the present invention a cell is contacted with at least one antibody or an antigen-binding portion thereof which binds to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, more preferably residues 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto, to result in the inhibition of cancerous growth of the cell as compared to an untreated cell. The method preferably results in the death of the cell.

The present invention also provides a method for inducing cell death of a cancer cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof which binds to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. In a preferred embodiment of the invention, at least one antibody or an antigen-binding portion thereof binds to an epitope contained within residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. The phrase "inducing cell death of a cancer cell" is taken to mean that that a cancer cell contacted with at least one antibody or an antigen-binding portion thereof which binds to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto, is caused to undergo cell necrosis, cell necrosis is associated with cellular disruption. Preferably, at least one antibody or an antigen-binding portion thereof binds to a polypeptide having a sequence comprising residues 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. Preferably,

the antibody or an antigen-binding portion thereof binds to a EphB4 protein having a sequence comprising residues 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody is Santa Cruz, polyclonal H antibody.

Cell death of a cancer cell may be assessed by a number of assays. For example, caspase-3 activation is considered to play a key role in the initiation of cellular events during cell death. Many different kits for the quantification of caspase-3 activity are available commercially. Mitochondrial membrane depolarization is often associated with the early stage of cell death. Changes in the membrane potential are presumed to be due to the opening of the mitochondrial permeability transition pores, which may play a central role in apoptosis. Depolarization can be detected by a number of different assays including the use of Rhodamine 123, a green-fluorescent cationic dye that accumulates in active mitochondria, which have high membrane potentials allowing quick and easy detection o cellular disruption. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant when the plasma membrane is damaged. LDH activity can easily be measured in culture supernatant by a single point assay using a spectrophotometric plate reader using commercially available kits. Elevated LDH in the culture medium is an indication of cell necrosis (death).

The morphology of a cell can also be examined to assess cell death. For instance, apoptosis is programmed cell death which is characterised by a series of typical morphological events, such as shrinkage of the cell and fragmentation into membrane-bound apoptotic bodies (19). These can be seen using a light microscope. In addition, a cell can be examined for the expression of genes related to cell death. In addition, RT-PCR analysis comparing EphB4 antibody treated and untreated cells from four different breast cancer cell lines has shown that *EphB4* gene expression is down-regulated in treated cells. RNA samples from four breast cancer cell lines (both treated and untreated) have been extracted for microarray analysis to identify other genes whose expression is also regulated by blocking the EphB4 signaling pathway. Changes to gene expression profiles can be confirmed by Northern analysis and RT-PCR.

A further aspect of the present invention is a method for treating or preventing cancer in a subject, the method comprising administering to the subject an effective amount of at least one antibody or an antigen-binding portion thereof which binds to a

polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. The method preferably results in the death of a cancer cell in the subject.

The cancer is preferably selected from the group consisting of breast cancer, prostate cancer, bowel cancer, colon cancer, ovarian cancer, lung cancer, melanoma, lymphoma and leukemia. The subject treated by the methods of the invention may be selected from, but is not limited to, the group consisting of humans, sheep, cattle, horses, bovine, pigs, poultry, dogs and cats.

In the method an effective amount of at least one antibody or an antigen-binding portion thereof which binds to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto, is administered to a subject. In a preferred embodiment of the invention, at least one antibody or an antigen-binding portion thereof binds to an epitope contained within residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, at least one antibody or an antigen-binding portion thereof binds to a polypeptide having a sequence comprising residues 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. The antibody or an antigen-binding portion thereof preferably binds to a EphB4 protein having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. The antibody or an antigen-binding portion thereof preferably specifically binds to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, more preferably residues 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the antibody or an antigen-binding portion thereof is a polyclonal or monoclonal antibody. Preferably, the antibody is Santa Cruz, polyclonal H-200 antibody.

The term "effective amount" means a dosage sufficient to provide treatment or prevention for the cancer being treated or prevented. This will vary depending on the subject and the type of cancer being effected. The effective amounts of at least one antibody or an antigen-binding portion thereof used in the methods of the present invention may vary depending upon the manner of administration, the condition of the animal to be treated, and ultimately will be decided by the attending scientist, physician or veterinarian. The amount of antibody or an antigen-binding portion thereof used to treat or prevent a subject will also vary depending upon the nature

and identity of the particular antibody or an antigen-binding portion thereof.

An antibody or an antigen-binding portion thereof is preferably administered to a subject by any suitable means known to those skilled in the art. Preferably, the antibody or an antigen-binding portion thereof can be introduced into a cell in numerous fashions, including, for example, microinjection of antibodies into a cell (17) or transforming hybridome mRNA encoding a desired antibody into a cell (18).

Preferably, the antibody or an antigen-binding portion thereof of the present invention is combined with a suitable pharmaceutically-acceptable carrier or diluent to form a pharmaceutical composition which may be suitable for administration to a human or animal subject. Suitable carriers or diluents include isotonic saline solutions, for example, phosphate-buffered saline. The pharmaceutical composition including at least one antibody or an antigen-binding portion thereof may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration. The antibody may be administered at a suitable dose dependent on the body weight of the subject. It is to be understood, however, that the routes of administration and dosages mentioned are intended to serve only as a guide since a person skilled in the art would be able to readily determine the optimum route of administration and dosage for any particular subject and cancer.

The antibody or an antigen-binding portion thereof used in the methods of the present invention may be combined with suitable excipients, such as emulsifiers, surfactants, stabilisers, dyes, penetration enhancers, anti-oxidants, water, salt solutions, alcohols, polyethylene glycols, gelatine, lactose, magnesium sterate and silicic acid. The antibody or an antigen-binding portion thereof is preferably formulated as a sterile aqueous solution. The antibody or an antigen-binding portion thereof can be combined with adjunct components that are compatible with the activity of the antibody. An antibody or an antigen-binding portion thereof used in the methods of the present invention may be preferably used to complement existing treatments for cancer. For example, the method of the present invention may also be used in combination with traditional cancer treatments such as radiotherapy, chemotherapy (eg using anthracyclines, 5FU, topoisomerase inhibitors, Cisplatin and Carboplatin), or hormone therapy or therapies utilising hormone modifiers (eg Catamoxifen).

In another aspect of the invention there is provided a method of identifying an agent which inhibits cancerous growth of a cell, the method comprising assessing the ability of the agent to bind to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. In a preferred embodiment of the invention, the agent binds to an epitope contained within residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the agent binds to a polypeptide having a sequence comprising residues 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the agent binds to a EphB4 protein having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto.

In the present specification the term "agent" is taken to include any molecule, compound or protein that can bind (interact with) residues 16 to 539 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. Suitable agents can preferably include an antibody or an antigen-binding portion thereof that binds to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1, more preferably residues 201 to 400 of SEQ ID NO: 1, or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the agent is an antibody or an antigen-binding portion thereof that is a polyclonal or monoclonal antibody. The method preferably comprises assessing the ability of the agent to induce cell death of a cancer cell. The agent is preferably a EphB4 ligand, such as an antibody or an antigen-binding portion thereof, that is preferably specific for EphB4 protein and may be developed or obtained commercially for testing in *in vitro* or *in vivo* systems for its ability to inhibit cancerous growth of a cell.

For instance, antibodies or antigen-binding portions thereof directed to specific epitopes of residues 16 to 539 of SEQ ID NO: 1 can be tested for their ability to inhibit cancerous growth of a cell and preferably induce cell death. Dose response curves to assess the IC50 of the antibodies can be conducted to test efficacy of each antibody tested. In addition, antibody/receptor-ligand binding studies can be performed to assess the ability of the antibody to prevent ligand binding. Tyrosine phosphorylation of the EphB4 receptor following antibody binding can be assessed by immunoprecipitation of the receptor with the respective antibody, followed by Western analysis with an anti-phosphotyrosine antibody to confirm that the EphB4 receptor is inactivated. The antibody with the best neutralising activity in terms of inhibiting tyrosine phosphorylation and cell growth *in vitro* and preventing ligand

binding to the EphB4 receptor at the lowest 50% inhibitory concentration (IC_{50}) can be selected for additional *in vivo* tests.

For instance, an *in vivo* model of metastasis and tumour growth using immune-deficient NOD-SCID (non-obese diabetic, combined immunodeficiency) mice can be used to test the ability of putative agents that can bind to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1 for their efficacy as an anti-cancer agent. Moreover, a diverse array of tumor cell lines that are available, most of which can be grown as xenografts, and these include the human breast cancer cell line MCF-7 and colon cancer cell line HT29, can be used for i*n vitro* testing. Xenograft tumours can be grown in the mouse model either after subcutaneous injection, where they will grow as a mass, or after injection into the tail vein allowing mimicry of the hematogenous spread of metastasis that results in secondary deposits in other organs. Once suitable engraftment periods and inoculation doses for each cell line have been established, the model can be used to test various agents,that bind to a polypeptide having a sequence comprising residues 16 to 539 of SEQ ID NO: 1. Cells may also be treated with sub-lethal doses of a chosen EphB4 antibody equating to the IC_{50} and IC_{75} to assess the engraftment of treated cells compared with non-treated cells. This will assess the effects of reduced functional expression of EphB4 on establishment and metastasis of tumour cell lines.

The *in vivo* models can also be used for pre-clinical assessment of potential new therapies for treatment of EphB4 positive tumours cell lines. The use of subcutaneous injection will allow the examination of tumours that have been allowed to establish for different periods of time. This can be used to determine the ability of an agent, such as an antibody or an antigen-binding portion thereof, to ablate newly and well-established tumours compared to vehicle control. The use of tail vein injections can be used to determine whether treatment with an antibody or an antigen-binding portion thereof will reduce number of metastases formed as a result of hematogenous spread. The agents identified by the methods of the present invention may be used for treatment or prevention of cancer. The present invention also provides an agent identified by the method described above.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The invention will hereinafter be described by way of the following non-limiting Figures and Examples.

Example 1 – Immunohistochemical localisation of EphB4:

An EphB4-specific polyclonal antibody was used to analyse the localisation of the EphB4 protein in tumour and normal tissue from the colon and breast showed marked increase in the levels of this protein in the tumour epithelial cells when compared with the matched normal tissue (as shown in Figure 1). The demonstration of high expression of *EphB4* on the tumour epithelial cells in two of the most commonly occurring cancers suggests that EphB4 is critical to the progression of these tumours.

Example 2 – RT-PCR expression of EphB4:

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to compare expression of *EphB4* (1187 bp) and internal 18S rRNA (489 bp) in five tumour (T) / normal (N) pairs (results shown in Figure 2). Analysis of 63 colon cancers from 60 patients indicated that *EphB4* is over-expressed in the tumour tissue of 80% of patients, implying broad application as a therapeutic target (Figure 2). The differential expression between tumour cells and normal tissue suggests anti-EphB4 tumour therapy may have a preferential effect on colon (and other) tumours.

A comparison of the expression profile of *EphB4* with that of other receptor protein tyrosine kinases already being targeted in clinical trials (HER2, EGFR and VEGFR) suggests that *EphB4* is expressed to a lesser degree in normal tissues. Information from EST databases suggests that low level expression of *EphB4* may be present in kidney, ovary and placenta, and very low level expression in heart, lung, peripheral nerves and vascular tissue. Accordingly therapies that target EphB4 may be expected to produce less side effects than those that target other receptor tyrosine kinases.

Example 3 - EphB4- specific antibody studies:

A direct tumoricidal effect of a EphB4-specific antibody *in vitro* was demonstrated. Incubation of the breast cancer cell line MCF-7 and the colon cancer cell line SW480 with three different concentrations of antibody (2 μg/ml, 1 μg/ml and 0.2 μg/ml) resulted in cell death in a dose dependent manner (see Figure 3). This effect was not

seen following exposure of the endothelial cell line HUVEC-C to the EphB4 antibody. Analysis of the caspase-3 activity suggested that cell death was not *via* apoptosis. Possible alternative mechanisms for the induction of cell death include ras-mediated non-apoptotic cell death () or restoration of gap junction intercellular communication (GJIC), a direct cell-cell communication pathway that is known to be prevented by Eph receptor signaling (21).

Polyclonalantibodies specific for EphB4 have been developed and are available commercially, for testing of these antibodies in *in vitro* and *in vivo* systems. Figure 4 show results of the percentage viability of breast cancer cells after treatment with selected EphB4 antibodies. Cell death effect is seen in treatment with the H-200antibody not related to complement activation (Figure 4).

The mechanism of cell death can be further by analysing changes to gene expression induced in cancer cells *in vitro* after incubation with sub-lethal doses of the EphB4 antibody using microarray techniques.

Example 4 - EphB4 antibody therapy:

Monoclonal antibody therapy can involve at least 15 mice being injected subcutaneously with 5×10^6 MCF-7 or HT29 cells. The tumour cells are then allowed to establish for 6 days. On the seventh day groups of 5 mice can be treated with either the EphB4 antibody or a human myeloma IgG2 control or phosphate-buffered saline vehicle (PBS) by intraperitoneal injection, twice a week for three weeks. Mice can then be euthenased after no more than 50 days (or, as in the case of the vehicle control, it is necessarily humane to do so) and the tumours resected and assessed for size, tumour cell death, and down-regulation of EphB4 by RT-PCR and western analysis. This will support the ability of the EphB4 antibody to prevent the growth of breast or colon tumours *in vivo*. Mice can again be injected subcutaneously with 5×10^6 MCF-7 or HT29 cells however this time treatment should begin after the tumours have been allowed to establish for different lengths of time (7, 11, 15 and 22 days after tumour implantation). The same treatments as used for the initial experiment will be used here and again the mice should be euthenased and the tumours resected for examination. Potential co-operative effects of the EphB4 antibody and chemotherapeutic agents such as cisplatin and doxorubicin may also be examined using similar *in vivo* experiments.

All publications discussed above are incorporated herein in their entirety.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this 16th day of September 2002

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Patent Attorneys for the Applicant:
BLAKE DAWSON WALDRON PATENT SERVICES

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Figure 1

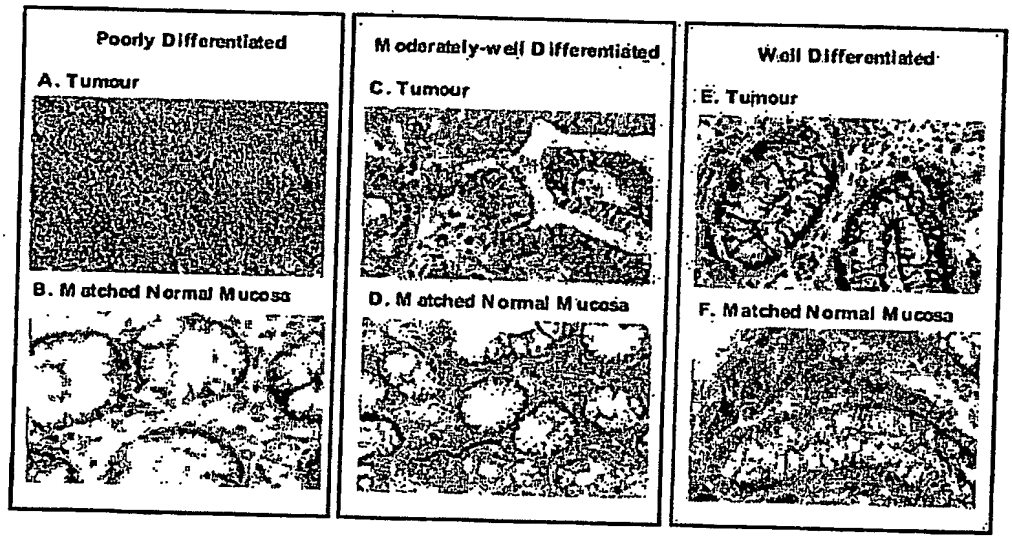
Poorly Differentiated
A. Tumour
B. Matched Normal Mucosa
Moderately-well Differentiated
C. Tumour
D. Matched Normal Mucosa
Well Differentiated
E. Tumour
F. Matched Normal Mucosa

Figure 2

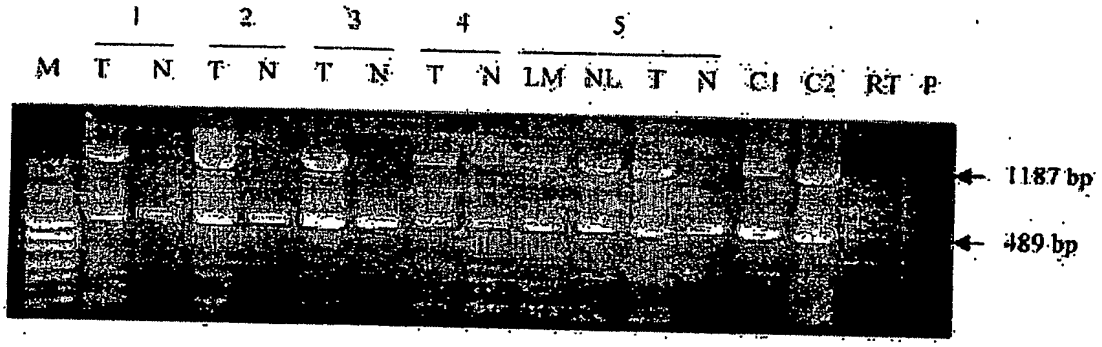

Figure 3

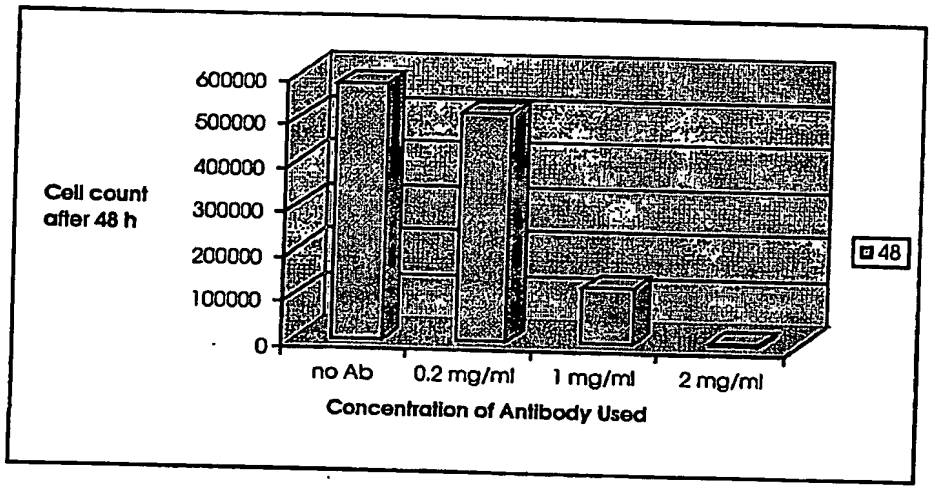
Cell count after 48 h
600000
500000
400000
300000
200000
100000
0
no Ab
0.2 mg/ml
1 mg/ml
2 mg/ml
Concentration of Antibody Used
48

Figure 4

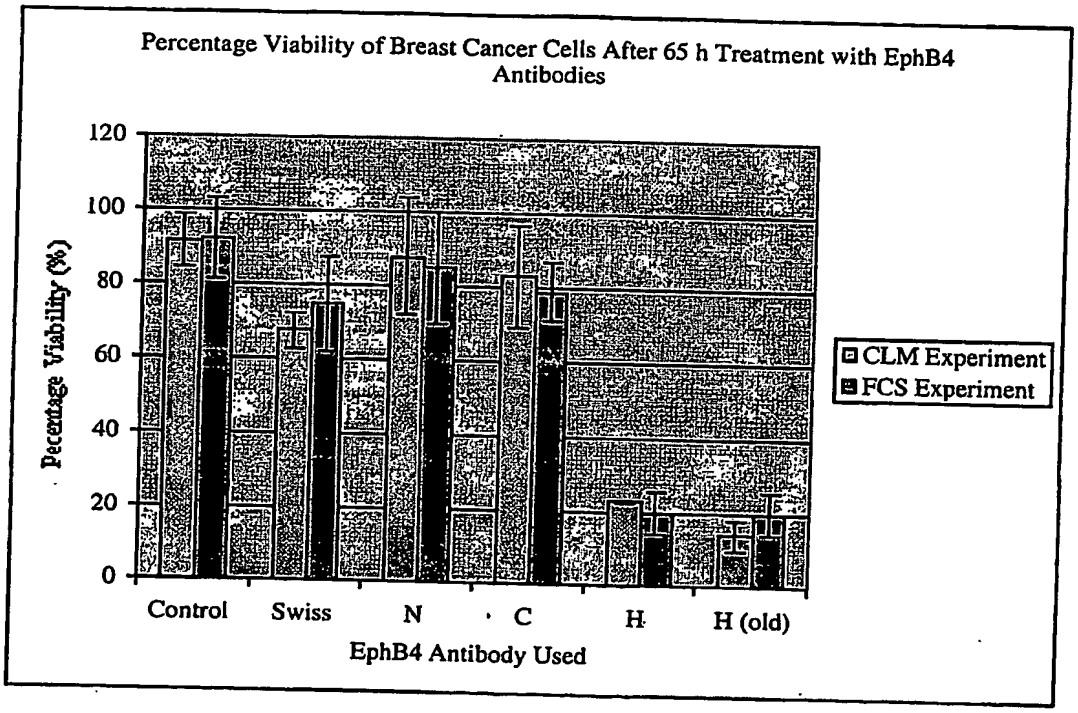
Percentage Viability of Breast Cancer Cells After 65 h Treatment with EphB4 Antibodies
Percentage Viability (%)
120
100
80
60
40
20
0
Control
Swiss
N
C
H
H (old)
EphB4 Antibody Used
CLM Experiment
FCS Experiment

Figure 5

SEQ ID NO:1

Homo Sapiens Ephrin type-B receptor 4 Precursor (EphB4)

1	MELRVLLCWA	SLAAALEETL	LNTKLETADL	KWVTFPQVDG	WEELSGLDE	EQHSVRTYEV
61	CDVQRAPGQA	HWLRTGWVPR	RGAVHVYATL	RFTMLECLSL	PRAGRSCKET	FTVFYYESDA
21	DTATALTPAW	MENPYIKVDT	VAAEHLTRKR	PGAEATGKVN	VKTLRLGPLS	KAGFYLAFQD
181	QGACMALLSL	HLFYKKCAQL	TVNLTRFPET	VPRELVVPVA	GSCVVDAVPA	PGPSPSLYCR
241	EDGQWAEQPV	TGCSCAPGFE	AAEGNTKCRA	CAQGTFKPLS	GEGSCQPCPA	NSHSNTIGSA
301	VCQCRVGYFR	ARTDPRGAPC	TTPPSAPRSV	VSRLNGSSLH	LEWSAPLESG	GREDLTYALR
361	CRECRPGGSC	APCGGDLTFD	PGPRDLVEPW	VVVRGLRPDF	TYTFEVTALN	GVSSLATGPV
421	PFEPVNVTTD	REVPPAVSDI	RVTRSSPSSL	SLAWAVPRAP	SGAVLDYEVK	HEKGAEGPS
481	SVRFLKTSEN	RAELRGLKRG	ASYLVQVRAR	SEAGYGPFGQ	EHHSQTQLDE	SEGWREQLAL
541	IAGTAVVGVV	LVLVVIVVAV	LCLRKQSNGR	EAEYSDKHGQ	YLIGHGTKVY	IDPFTYEDPN
601	EAVREFAKEI	DVSYVKIEEV	IGAGEFGEVC	RGRLKAPGKK	ESCVAIKTLK	GGYTERQRRE
661	FLSEASIMGQ	FEHPNIIRLE	GVVTNSMPVM	ILTEFMENGA	LDSFLRLNDG	QFTVIQLVGM
721	LRGIASGMRY	LAEMSYVHRD	LAARNILVNS	NLVCKVSDFG	LSRFLEENSS	DPTYTSSLGG
781	KIPIRWTAPE	AIAFRKFTSA	SDAWSYGIVM	WEVMSFGERP	YWDMSNQDVI	NAIEQDYRLP
841	PPPDCPTSLH	QLMLDCWQKD	RNARPRFPQV	VSALDKMIRN	PASLKIVARE	GGASHPLLD
901	QRQPHYSAFG	SVGEWLRAIK	MGRYEESFAA	AGFGSFELVS	QISAEDLLRI	GVTLAGHQKK
961	ILASVQHMKS	QAKPGTPGGT	GGPAPQY			